

## A PROCESS OF PREPARING AN ANTI-OXIDANT

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> CROSS-Reference to Related Applications is a 371 of  
This application claims the benefit of and priority to PCT application PCT/IB 98/00708  
filed May 06, 1998 designating the U.S., which claims priority from Great Britain Patent  
Application 9709161.5  
The present invention relates to a process of preparing an anti-oxidant. filed May 06, 1997

5 An anti-oxidant prevents, inhibits or reduces the oxidation rate of an oxidisable medium. In particular, anti-oxidants are used for the preservation of food, especially when the food is or comprises a fat. Typical chemical anti-oxidants include aromatic amines, substituted phenols and sulphur compounds. Examples of anti-oxidants for food products are polyvinylpolypyrrolidone, dithiothreitol, sulphur dioxide, synthetic  
10  $\gamma$ -tocopherol,  $\delta$ -tocopherol, L-ascorbic acid, sodium L-ascorbate, calcium L-ascorbate, ascorbyl palmitate, propyl gallate, octyl gallate, dodecyl gallate, lecithin, diphenylamine ethoxyquin and butylated hydroxytoluene. Two commonly used anti-oxidants are GRINDOX 142 (obtained from Danisco A/S) and GRINDOX 1029 (obtained from Danisco A/S).

15 Typically, anti-oxidants are added to foodstuffs, such as beverages.

For example, anti-oxidants are used in the preparation of alcoholic beverages such as beer, cider, ale etc.. In particular, there is a wide spread use of anti-oxidants in the  
20 preparation of wine. In this regard, Butzke and Bisson in Agro-Food-Industry Hi-Tech (July/August 1996 pages 26-30) present a review of wine manufacture.

According to Butzke and Bisson (*ibid*):

25 "Wine is the product of the natural fermentation of grape must or juice. In the case of red wine, the skins are present during the initial fermentation to allow extraction of pigment and important flavour and aroma constituents from the skin. The term "must" refers to the crushed whole grapes. In the case of white wine production, skins are  
30 removed prior to fermentation and only the juice is retained and processed. ....

*Grapes are harvested and brought directly to the winery from the field. The grapes are then crushed at the winery and the must either transferred to a tank for fermentation (red wine) or pressed to separate juice from the skin and seeds (white wine). In this latter case, the juice is then transferred to a tank for fermentation. The tanks may either be inoculated with a commercial wine strain of Saccharomyces or allowed to undergo a natural or uninoculated fermentation. In a natural fermentation, Saccharomyces cells are greatly outnumbered by wild (non-Saccharomyces) yeast and bacteria at the beginning of fermentation. By the end of the fermentation Saccharomyces is the dominant and most often only organism isolateable. Inoculation with a commercial wine strain or with fermenting juice or must changes the initial ratio of the numbers of different microorganisms, allowing Saccharomyces to dominate the fermentation much earlier.*

*The metabolic activity of microorganisms in wine results in the production of aroma and flavour compounds some of which are highly objectionable to the consumer and all of which are distinct from the compounds responsible for the varietal character of the wine. .... Sulphur dioxide addition prevents chemical oxidation reactions and in this sense is an important stabilizer of the natural grape aroma and flavour. It may be added to the must or juice to preserve flavour, not necessarily as an antimicrobial agent. However, its antimicrobial activity must be considered when choosing a strain to be genetically modified for wine production."*

Hence, potentially harmful chemicals - such as sulphur dioxide - are used in wine manufacture.

The present invention seeks to overcome any problems associated with the prior art methods of preparing foodstuffs with antioxidants.

According to a first aspect of the present invention there is provided a process of preparing a medium that comprises an anti-oxidant and at least one other component, the process comprising preparing *in situ* in the medium the anti-oxidant; and wherein the anti-oxidant is prepared from a glucan by use of recombinant DNA techniques.

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According to a second aspect of the present invention there is provided a process of preparing a medium that comprises an anti-oxidant and at least one other component, the process comprising preparing *in situ* in the medium the anti-oxidant; and wherein the anti-oxidant is prepared by use of a recombinant glucan lyase.

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According to a third aspect of the present invention there is provided a medium prepared by the process according to the present invention.

Other aspects of the present invention include:

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Use of anhydrofructose as an anti-oxidant for a medium comprising at least one other component, wherein the anhydrofructose is prepared *in situ* in the medium.

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Use of anhydrofructose as a means for imparting or improving stress tolerance in a plant, wherein the anhydrofructose is prepared *in situ* in the plant.

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Use of anhydrofructose as a means for imparting or improving the transformation of a grape, wherein the anhydrofructose is prepared *in situ* in the grape.

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Use of anhydrofructose as a means for increasing antioxidant levels in a foodstuff (preferably a fruit or vegetable, more preferably a fresh fruit or a fresh vegetable), wherein the anhydrofructose is prepared *in situ* in the foodstuff.

Use of anhydrofructose as a pharmaceutical in a foodstuff, wherein the anhydrofructose is prepared *in situ* in the foodstuff.

5 A method of administering a foodstuff comprising anhydrofructose, wherein the anhydrofructose is in a pharmaceutically acceptable amount and acts as a pharmaceutical; and wherein the anhydrofructose has been prepared *in situ* in the foodstuff.

10 Use of anhydrofructose as a nutraceutical in a foodstuff, wherein the anhydrofructose is prepared *in situ* in the foodstuff.

15 A method of administering a foodstuff comprising anhydrofructose, wherein the anhydrofructose is in a nutraceutically acceptable amount and acts as a nutraceutical; and wherein the anhydrofructose has been prepared *in situ* in the foodstuff.

Use of glucan lyase as a means for imparting or improving stress tolerance in a plant, wherein the glucan lyase is prepared *in situ* in the plant.

20 Use of glucan lyase as a means for imparting or improving the transformation of a grape, wherein the glucan lyase is prepared *in situ* in the grape.

25 Use of glucan lyase as a means for increasing antioxidant levels in a foodstuff (preferably a fruit or vegetable, more preferably a fresh fruit or a fresh vegetable), wherein the glucan lyase is prepared *in situ* in the foodstuff.

Use of glucan lyase in the preparation of a pharmaceutical in a foodstuff, wherein the glucan lyase is prepared *in situ* in the foodstuff.

30 A method of administering a foodstuff comprising an antioxidant, wherein the antioxidant is in a pharmaceutically acceptable amount and acts as a pharmaceutical; and wherein the antioxidant has been prepared *in situ* in the

foodstuff from a glucan lyase.

Use of glucan lyase in the preparation of a nutraceutical in a foodstuff, wherein the glucan lyase is prepared *in situ* in the foodstuff.

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A method of administering a foodstuff comprising an antioxidant, wherein the antioxidant is in a nutraceutically acceptable amount and acts as a nutraceutical; and wherein the antioxidant has been prepared *in situ* in the foodstuff from a glucan lyase.

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Use of a nucleotide sequence coding for a glucan lyase as a means for imparting or improving stress tolerance in a plant, wherein the nucleotide sequence is expressed *in situ* in the plant.

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Use of a nucleotide sequence coding for a glucan lyase as a means for imparting or improving the transformation of a grape, wherein the nucleotide sequence is expressed *in situ* in the grape.

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Use of a nucleotide sequence coding for a glucan lyase as a means for increasing antioxidant levels in a foodstuff (preferably a fruit or vegetable, more preferably a fresh fruit or a fresh vegetable), wherein the nucleotide sequence is expressed *in situ* in the foodstuff.

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Use of a nucleotide sequence coding for a glucan lyase as a means for creating a pharmaceutical in a foodstuff, wherein the nucleotide sequence is expressed *in situ* in the foodstuff.

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A method of administering a foodstuff comprising an antioxidant, wherein the antioxidant is in a pharmaceutically acceptable amount and acts as a pharmaceutical; and wherein the antioxidant has been prepared *in situ* in the foodstuff by means of a nucleotide sequence coding for a glucan lyase.

Use of a nucleotide sequence coding for a glucan lyase as a means for creating a nutraceutical in a foodstuff, wherein the nucleotide sequence is expressed *in situ* in the foodstuff.

5 A method of administering a foodstuff comprising an antioxidant, wherein the antioxidant is in a nutraceutically acceptable amount and acts as a nutraceutical; and wherein the antioxidant has been prepared *in situ* in the foodstuff by means of a nucleotide sequence coding for a glucan lyase.

10 The term "nutraceutical" means a compound that is capable of acting as a nutrient (i.e. it is suitable for, for example, oral administration) as well as being capable of exhibiting a pharmaceutical effect and/or cosmetic effect.

In contrast to the usual practice of adding anti-oxidants media, such as foodstuffs, we  
15 have now found that particular anti-oxidants can be prepared *in situ* in the medium.

The *in situ* preparation of anti-oxidants is particularly advantageous in that less, or even no, additional anti-oxidants need be added to the medium, such as a food  
20 product.

The present invention is also believed to be advantageous as it provides a means of improving stress tolerance of plants.

The present invention is also advantageous as it provides a means for viably  
25 transforming grape.

The present invention is further advantageous in that it enables the levels of antioxidants in foodstuffs to be elevated. This may have beneficial health implications. In this regard, recent reports (e.g. Biotechnology Newswatch April 21  
30 1997 "*Potent Antioxidants, as strong as those in fruit, found in coffee*" by Marjorie Shaffer) suggest that antioxidants have a pharmaceutical benefit, for example in preventing or suppressing cancer formation.

General *in situ* preparation of antioxidants in plants has been previously reviewed by Badiani *et al* in Agro-Food-Industry Hi-Tech (March/April 1996 pages 21-26). It is to be noted, however, that this review does not mention preparing *in situ* antioxidants from a glucan, let alone by use of a recombinant glucan lyase.

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Preferably, the glucan comprises  $\alpha$ -1,4 links.

Preferably, the glucan is starch or a unit of starch.

- 10 Preferably, the glucan is a substrate for a recombinant enzyme such that contact of the glucan with the recombinant enzyme yields the anti-oxidant.

Preferably, the enzyme is a glucan lyase.

- 15 Preferably, the enzyme is an  $\alpha$ -1,4-glucan lyase.

Preferably, the enzyme comprises any one of the sequences shown as SEQ ID Nos 1-6, or a variant, homologue or fragment thereof.

- 20 Preferably, the enzyme is any one of the sequences shown as SEQ ID Nos 1-6.

Preferably, the enzyme is encoded by a nucleotide sequence comprising any one of the sequences shown as SEQ ID Nos 7-12, or a variant, homologue or fragment thereof.

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Preferably, the enzyme is encoded by a nucleotide sequence having any one of the sequences shown as SEQ ID Nos 7-12.

Preferably, the anti-oxidant is anhydrofructose.

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Preferably, the anti-oxidant is 1,5-D-anhydrofructose.

Preferably, the medium is, or is used in the preparation of, a foodstuff.

Preferably, the foodstuff is a beverage.

5 Preferably, the beverage is an alcoholic beverage.

Preferably, the beverage is a wine.

10 Preferably, the anti-oxidant is prepared *in situ* in the component and is then released into the medium.

Preferably, the component is a plant or a part thereof.

Preferably, the component is all or part of a cereal or a fruit.

15 Preferably, the component is all or part of a grape.

20 The medium may be used as or in the preparation of a foodstuff, which includes beverages. In the alternative, the medium may be for use in polymer chemistry. In this regard, the *in situ* generated anti-oxidants could therefore act as oxygen scavengers during, for example, the synthesis of polymers, such as the synthesis of bio-degradable plastic.

25 In accordance with the present invention, the anti-oxidant (preferably anhydrofructose) is prepared *in situ* in the medium. In other words, the antioxidant (preferably anhydrofructose) that is prepared *in situ* in the medium is used as an anti-oxidant in the medium. In one embodiment, the antioxidant (preferably anhydrofructose) that is prepared *in situ* in the medium is used as the main anti-oxidant in the medium.

30 The term "*in situ* in the medium" as used herein includes the anti-oxidant being prepared by action of a recombinant enzyme expressed by the component on a glucan - which glucan is a substrate for the enzyme. The term also includes the anti-oxidant

being prepared by action of a recombinant enzyme expressed by the component on a glucan - which glucan is a substrate for the enzyme - within the component and the subsequent generation of the anti-oxidant. The term also includes the recombinant enzyme being expressed by the component and then being released into the medium, which enzyme acts on a glucan - which glucan is a substrate for the enzyme - present in the medium to form the anti-oxidant in the medium. The term also covers the presence or addition of another component to the medium, which component then expresses a recombinant nucleotide sequence which results in exposure of part or all of the medium to an anti-oxidant, which anti-oxidant may be a recombinant enzyme or a recombinant protein expressed and released by the other component, or it may be a product of a glucan - which glucan is a substrate for the enzyme - within the medium that has been exposed to the recombinant enzyme or the recombinant protein.

The term "by use of recombinant DNA techniques" as used herein includes the anti-oxidant being any obtained by use of a recombinant enzyme or a recombinant protein, which enzyme or protein acts on the glucan. The term also includes the anti-oxidant being any obtained by use of an enzyme or protein, which enzyme or protein acts on a recombinant glucan.

The term "starch" in relation to the present invention includes native starch, degraded starch, modified starch, including its components amylose and amylopectin, and the glucose units thereof.

The terms "variant", "homologue" or "fragment" in relation to the enzyme include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acid from or to the sequence providing the resultant amino acid sequence has  $\alpha$ -glucan lyase activity, preferably having at least the same activity of any one of the enzymes shown as SEQ ID No. 1-6. In particular, the term "homologue" covers homology with respect to structure and/or function providing the resultant enzyme has  $\alpha$ -glucan lyase activity. With respect to sequence homology, preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to any one of the sequences shown as SEQ ID No.s 1-6. More

preferably there is at least 95 %, more preferably at least 98 %, homology to any one of the sequences shown as SEQ ID No. 1-6.

5 The terms "variant", "homologue" or "fragment" in relation to the nucleotide sequence coding for the enzyme include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid from or to the sequence providing the resultant nucleotide sequence codes for an enzyme having  $\alpha$ -glucan lyase activity, preferably having at least the same activity of any one of the enzymes shown as SEQ ID No. 1-6. In particular, the term "homologue" covers  
10 homology with respect to structure and/or function providing the resultant nucleotide sequence codes for an enzyme having  $\alpha$ -glucan lyase activity. With respect to sequence homology, preferably there is at least 75 %, more preferably at least 85 %, more preferably at least 90 % homology to any one of the sequences shown as SEQ ID No. 7-12. More preferably there is at least 95 %, more preferably at least 98 %,   
15 homology to any one of the sequences shown as SEQ ID No. 7-12.

The above terms are synonymous with allelic variations of the sequences.

20 The present invention also covers nucleotide sequences that can hybridise to the nucleotide sequence of the present invention.

The term "nucleotide" in relation to the present invention includes cDNA.

25 According to the present invention there is therefore provided a method of preparing *in situ* in an oxidisable medium an anti-oxidant. In a preferred embodiment, the anti-oxidant is anhydrofructose, more preferably 1,5-D-anhydrofructose. 1,5-D-anhydrofructose has been chemically synthesised (Lichtenthaler in Tetrahedron Letters Vol 21 pp 1429-1432). 1,5-D-anhydrofructose is further discussed in WO 95/10616, WO 95/10618 and GB-B-2294048.

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The main advantages of using 1,5-D-anhydrofructose as an anti-oxidant are that it is a natural product, it is non-metabolisable, it is easy to manufacture, it is water-soluble, and it is generally non-toxic.

5 According to WO 95/10616, WO 95/10618 and GB-B-2294048, 1,5-D-anhydrofructose may be prepared by the enzymatic modification of substrates based on  $\alpha$ -1,4-glucan by use of the enzyme  $\alpha$ -1,4-glucan lyase. A typical  $\alpha$ -1,4-glucan based substrate is starch.

10 Today, starches have found wide uses in industry mainly because they are cheap raw materials. There are many references in the art to starch. For example, starch is discussed by Salisbury and Ross in Plant Physiology (Fourth Edition, 1991, Published by Wadsworth Publishing Company - especially section 11.7). In short, however, starch is one of the principal energy reserves of plants. It is often found in colourless  
15 plastids (amyloplasts), in storage tissue and in the stroma of chloroplasts in many plants. Starch is a polysaccharide carbohydrate. It comprises two main components: amylose and/or amylopectin. Both amylose and/or amylopectin consist of straight chains of  $\alpha$ (1,4)-linked glucose units (ie glycosyl residues) but in addition amylopectin includes  $\alpha$ (1,6) branched glucose units.

20 Some of the glucan lyases discussed in WO 95/10616 and WO 95/10618 that are suitable for producing 1,5-D-anhydrofructose from starch are shown as SEQ I.D. No.s 1-4. Some of the glucan lyases discussed in GB-B-2294048 that are suitable for producing 1,5-D-anhydrofructose from starch are shown as SEQ I.D. No.s 5-6.

25 Some of the nucleotide sequences coding for glucan lyases discussed in WO 95/10616 and WO 95/10618 that are suitable for producing 1,5-D-anhydrofructose from starch are shown as SEQ I.D. No.s 7-10. Some of the nucleotide sequences coding for glucan lyases discussed in GB-B-2294048 that are suitable for producing 1,5-D-  
30 anhydrofructose from starch are shown as SEQ I.D. No.s 11-12.

A further glucan lyase is discussed in WO 94/09122.

The recombinant nucleotide sequences coding for the enzyme may be cloned from sources such as a fungus, preferably *Morchella costata* or *Morchella vulgaris*, or from a fungally infected algae, preferably *Gracilariopsis lemaneiformis*, or from algae lone, preferably *Gracilariopsis lemaneiformis*.

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In a preferred embodiment, the 1,5-D-anhydrofructose is prepared *in situ* by treating an  $\alpha$ -1,4-glucan with a recombinant  $\alpha$ -1,4-glucan lyase, such as any one of those presented as SEQ I.D. No.s 1-6.

- 10 Detailed commentary on how to prepare the enzymes shown as sequences SEQ I.D. No.s 1-6 may be found in the teachings of WO 95/10616, WO 95/10618 and GB-B-2294048. Likewise, detailed commentary on how to isolate and clone the nucleotide sequences SEQ I.D. No.s 7-12 may be found in the teachings of WO 95/10616, WO 95/10618 and GB-B-2294048.

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If the glucan contains links other than and in addition to the  $\alpha$ -1,4- links the recombinant  $\alpha$ -1,4-glucan lyase can be used in conjunction with a suitable reagent that can break the other links - such as a recombinant hydrolase - preferably a recombinant glucanohydrolase.

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General teachings of recombinant DNA techniques may be found in Sambrook, J., Fritsch, E.F., Maniatis T. (Editors) Molecular Cloning. A laboratory manual. Second edition. Cold Spring Harbour Laboratory Press. New York 1989.

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In order to express a nucleotide sequence, the host organism can be a prokaryotic or a eukaryotic organism. Examples of suitable prokaryotic hosts include *E. coli* and *Bacillus subtilis*. Teachings on the transformation of prokaryotic hosts is well documented in the art, for example see Sambrook *et al* (Molecular Cloning: A Laboratory Manual, 2nd edition, 1989, Cold Spring Harbor Laboratory Press). If a

30 prokaryotic host is used then the gene may need to be suitably modified before transformation - such as by removal of introns.

In one embodiment, the host organism can be of the genus *Aspergillus*, such as *Aspergillus niger*. A transgenic *Aspergillus* can be prepared by following the teachings of Rambosek, J. and Leach, J. 1987 (Recombinant DNA in filamentous fungi: Progress and Prospects. CRC Crit. Rev. Biotechnol. 6:357-393), Davis R.W. 1994 (Heterologous gene expression and protein secretion in *Aspergillus*. In: Martinelli S.D., Kinghorn J.R.( Editors) *Aspergillus: 50 years on. Progress in industrial microbiology* vol 29. Elsevier Amsterdam 1994. pp 525-560), Ballance, D.J. 1991 (Transformation systems for Filamentous Fungi and an Overview of Fungal Gene structure. In: Leong, S.A., Berka R.M. (Editors) *Molecular Industrial Mycology. Systems and Applications for Filamentous Fungi*. Marcel Dekker Inc. New York 1991. pp 1-29) and Turner G. 1994 (Vectors for genetic manipulation. In: Martinelli S.D., Kinghorn J.R.( Editors) *Aspergillus: 50 years on. Progress in industrial microbiology* vol 29. Elsevier Amsterdam 1994. pp. 641-666). However, the following commentary provides a summary of those teachings for producing transgenic *Aspergillus*.

For almost a century, filamentous fungi have been widely used in many types of industry for the production of organic compounds and enzymes. For example, traditional japanese koji and soy fermentations have used *Aspergillus sp.* Also, in this century *Aspergillus niger* has been used for production of organic acids particular citric acid and for production of various enzymes for use in industry.

There are two major reasons why filamentous fungi have been so widely used in industry. First filamentous fungi can produce high amounts of extracellular products, for example enzymes and organic compounds such as antibiotics or organic acids. Second filamentous fungi can grow on low cost substrates such as grains, bran, beet pulp etc. The same reasons have made filamentous fungi attractive organisms as hosts for heterologous expression of recombinant enzymes according to the present invention.

In order to prepare the transgenic *Aspergillus*, expression constructs are prepared by inserting a requisite nucleotide sequence into a construct designed for expression in filamentous fungi.

- 5 Several types of constructs used for heterologous expression have been developed. These constructs can contain a promoter which is active in fungi. Examples of promoters include a fungal promoter for a highly expressed extracellular enzyme, such as the glucoamylase promoter or the  $\alpha$ -amylase promoter. The nucleotide sequence can be fused to a signal sequence which directs the protein encoded by the nucleotide  
10 sequence to be secreted. Usually a signal sequence of fungal origin is used. A terminator active in fungi ends the expression system.

- Another type of expression system has been developed in fungi where the nucleotide sequence can be fused to a smaller or a larger part of a fungal gene encoding a stable  
15 protein. This can stabilize the protein encoded by the nucleotide sequence. In such a system a cleavage site, recognized by a specific protease, can be introduced between the fungal protein and the protein encoded by the nucleotide sequence, so the produced fusion protein can be cleaved at this position by the specific protease thus liberating the protein encoded by the nucleotide sequence. By way of example, one  
20 can introduce a site which is recognized by a KEX-2 like peptidase found in at least some *Aspergilli*. Such a fusion leads to cleavage *in vivo* resulting in protection of the expressed product and not a larger fusion protein.

- Heterologous expression in *Aspergillus* has been reported for several genes coding for  
25 bacterial, fungal, vertebrate and plant proteins. The proteins can be deposited intracellularly if the nucleotide sequence is not fused to a signal sequence. Such proteins will accumulate in the cytoplasm and will usually not be glycosylated which can be an advantage for some bacterial proteins. If the nucleotide sequence is equipped with a signal sequence the protein will accumulate extracellularly.

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With regard to product stability and host strain modifications, some heterologous proteins are not very stable when they are secreted into the culture fluid of fungi.

Most fungi produce several extracellular proteases which degrade heterologous proteins. To avoid this problem special fungal strains with reduced protease production have been used as host for heterologous production.

5 For the transformation of filamentous fungi, several transformation protocols have been developed for many filamentous fungi (Ballance 1991, *ibid*). Many of them are based on preparation of protoplasts and introduction of DNA into the protoplasts using PEG and  $\text{Ca}^{2+}$  ions. The transformed protoplasts then regenerate and the transformed fungi are selected using various selective markers. Among the markers  
10 used for transformation are a number of auxotrophic markers such as *argB*, *trpC*, *niaD* and *pyrG*, antibiotic resistance markers such as benomyl resistance, hygromycin resistance and phleomycin resistance. A commonly used transformation marker is the *amdS* gene of *A. nidulans* which in high copy number allows the fungus to grow with acrylamide as the sole nitrogen source.

15 In another embodiment the transgenic organism can be a yeast. In this regard, yeast have also been widely used as a vehicle for heterologous gene expression. The species *Saccharomyces cerevisiae* has a long history of industrial use, including its use for heterologous gene expression. Expression of heterologous genes in  
20 *Saccharomyces cerevisiae* has been reviewed by Goodey *et al* (1987, Yeast Biotechnology, D R Berry *et al*, eds, pp 401-429, Allen and Unwin, London) and by King *et al* (1989, Molecular and Cell Biology of Yeasts, E F Walton and G T Yarronton, eds, pp 107-133, Blackie, Glasgow).

25 For several reasons *Saccharomyces cerevisiae* is well suited for heterologous gene expression. First, it is non-pathogenic to humans and it is incapable of producing certain endotoxins. Second, it has a long history of safe use following centuries of commercial exploitation for various purposes. This has led to wide public acceptability. Third, the extensive commercial use and research devoted to the  
30 organism has resulted in a wealth of knowledge about the genetics and physiology as well as large-scale fermentation characteristics of *Saccharomyces cerevisiae*.

A review of the principles of heterologous gene expression in *Saccharomyces cerevisiae* and secretion of gene products is given by E Hinchcliffe E Kenny (1993, "Yeast as a vehicle for the expression of heterologous genes", Yeasts, Vol 5, Anthony H Rose and J Stuart Harrison, eds, 2nd edition, Academic Press Ltd.).

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Several types of yeast vectors are available, including integrative vectors, which require recombination with the host genome for their maintenance, and autonomously replicating plasmid vectors.

- 10 In order to prepare the transgenic *Saccharomyces*, expression constructs are prepared by inserting the nucleotide sequence into a construct designed for expression in yeast. Several types of constructs used for heterologous expression have been developed. The constructs contain a promoter active in yeast fused to the nucleotide sequence, usually a promoter of yeast origin, such as the GAL1 promoter, is used. Usually a  
15 signal sequence of yeast origin, such as the sequence encoding the SUC2 signal peptide, is used. A terminator active in yeast ends the expression system.

- For the transformation of yeast several transformation protocols have been developed. For example, a transgenic *Saccharomyces* can be prepared by following the teachings  
20 of Hinnen *et al* (1978, Proceedings of the National Academy of Sciences of the USA 75, 1929); Beggs, J D (1978, Nature, London, 275, 104); and Ito, H *et al* (1983, J Bacteriology 153, 163-168).

- The transformed yeast cells are selected using various selective markers. Among the  
25 markers used for transformation are a number of auxotrophic markers such as LEU2, HIS4 and TRP1, and dominant antibiotic resistance markers such as aminoglycoside antibiotic markers, eg G418.

- Another host organism is a plant. In this regard, the art is replete with references for  
30 preparing transgenic plants. Two documents that provide some background commentary on the types of techniques that may be employed to prepare transgenic plants are EP-B-0470145 and CA-A-2006454 - some of which commentary is

presented below.

The basic principle in the construction of genetically modified plants is to insert genetic information in the plant genome so as to obtain a stable maintenance of the inserted genetic material.

Several techniques exist for inserting the genetic information, the two main principles being direct introduction of the genetic information and introduction of the genetic information by use of a vector system. A review of the general techniques may be found in articles by Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27).

Thus, in one aspect, the present invention relates to a vector system which carries a recombinant nucleotide sequence and which is capable of introducing the nucleotide sequence into the genome of an organism, such as a plant, and wherein the nucleotide sequence is capable of preparing *in situ* an anti-oxidant.

The vector system may comprise one vector, but it can comprise at least two vectors. In the case of two vectors, the vector system is normally referred to as a binary vector system. Binary vector systems are described in further detail in Gynheung An et al. (1980), Binary Vectors, *Plant Molecular Biology Manual A3*, 1-19.

One extensively employed system for transformation of plant cells with a given promoter or nucleotide sequence or construct is based on the use of a Ti plasmid from *Agrobacterium tumefaciens* or a Ri plasmid from *Agrobacterium rhizogenes* (An et al. (1986), *Plant Physiol.* 81, 301-305 and Butcher D.N. et al. (1980), *Tissue Culture Methods for Plant Pathologists*, eds.: D.S. Ingrams and J.P. Helgeson, 203-208).

Several different Ti and Ri plasmids have been constructed which are suitable for the construction of the plant or plant cell constructs described above.

The nucleotide sequence of the present invention should preferably be inserted into

the Ti-plasmid between the border sequences of the T-DNA or adjacent a T-DNA sequence so as to avoid disruption of the sequences immediately surrounding the T-DNA borders, as at least one of these regions appear to be essential for insertion of modified T-DNA into the plant genome.

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As will be understood from the above explanation, if the organism is a plant, then the vector system of the present invention is preferably one which contains the sequences necessary to infect the plant (e.g. the *vir* region) and at least one border part of a T-DNA sequence, the border part being located on the same vector as the genetic construct. Preferably, the vector system is an *Agrobacterium tumefaciens* Ti-plasmid or an *Agrobacterium rhizogenes* Ri-plasmid or a derivative thereof, as these plasmids are well-known and widely employed in the construction of transgenic plants, many vector systems exist which are based on these plasmids or derivatives thereof.

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In the construction of a transgenic plant the nucleotide sequence or construct or vector of the present invention may be first constructed in a microorganism in which the vector can replicate and which is easy to manipulate before insertion into the plant. An example of a useful microorganism is *E. coli*., but other microorganisms having the above properties may be used. When a vector of a vector system as defined above has been constructed in *E. coli*. it is transferred, if necessary, into a suitable *Agrobacterium* strain, e.g. *Agrobacterium tumefaciens*. The Ti-plasmid harbouring the first nucleotide sequence or construct of the invention is thus preferably transferred into a suitable *Agrobacterium* strain, e.g. *A. tumefaciens*, so as to obtain an *Agrobacterium* cell harbouring the promoter or nucleotide sequence or construct of the invention, which DNA is subsequently transferred into the plant cell to be modified.

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As reported in CA-A-2006454, a large number of cloning vectors are available which contain a replication system in *E. coli* and a marker which allows a selection of the transformed cells. The vectors contain for example pBR322, the pUC series, the M13 mp series, pACYC 184 etc. In this way, the promoter or nucleotide or construct of the present invention can be introduced into a suitable restriction position

in the vector. -The contained plasmid is used for the transformation in *E.coli*. The *E.coli* cells are cultivated in a suitable nutrient medium and then harvested and lysed. The plasmid is then recovered and then analysed - such as by any one or more of the following techniques: sequence analysis, restriction analysis, electrophoresis and further biochemical-molecular biological methods. After each manipulation, the used DNA sequence can be restricted or selectively amplified by PCR techniques and connected with the next DNA sequence. Each sequence can be cloned in the same or different plasmid.

After each introduction method of the nucleotide sequence or construct or vector according to the present invention in the plants the presence and/or insertion of further DNA sequences may be necessary. If, for example, for the transformation the Ti- or Ri-plasmid of the plant cells is used, at least the right boundary and often however the right and the left boundary of the Ti- and Ri-plasmid T-DNA, as flanking areas of the introduced genes, can be connected. The use of T-DNA for the transformation of plant cells has been intensively studied and is described in EP-A-120516; Hoekema, in: The Binary Plant Vector System Offset-drukkerij Kanters B.B., Alblasterdam, 1985, Chapter V; Fraley, *et al.*, Crit. Rev. Plant Sci., 4:1-46; and An *et al.*, EMBO J. (1985) 4:277-284.

Direct infection of plant tissues by *Agrobacterium* is a simple technique which has been widely employed and which is described in Butcher D.N. *et al.* (1980), *Tissue Culture Methods for Plant Pathologists*, eds.: D.S. Ingrams and J.P. Helgeson, 203-208. For further teachings on this topic see Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27). With this technique, infection of a plant may be done on a certain part or tissue of the plant, i.e. on a part of a leaf, a root, a stem or another part of the plant.

Typically, with direct infection of plant tissues by *Agrobacterium* carrying the first nucleotide sequence or the construct, a plant to be infected is wounded, e.g. by cutting the plant with a razor or puncturing the plant with a needle or rubbing the

plant with an abrasive. The wound is then inoculated with the *Agrobacterium*. The inoculated plant or plant part is then grown on a suitable culture medium.

When plant cells are constructed, these cells are grown and, optionally, maintained  
5 in a medium according to the present invention following well-known tissue culturing methods - such as by culturing the cells in a suitable culture medium supplied with the necessary growth factors such as amino acids, plant hormones, vitamins, etc, but wherein the culture medium comprises a component according to the present invention. Regeneration of the transformed cells into genetically modified plants may  
10 be accomplished using known methods for the regeneration of plants from cell or tissue cultures, for example by selecting the transformed shoots and by subculturing the shoots on a medium containing the appropriate nutrients, plant hormones, etc.

Further teachings on plant transformation may be found in EP-A-0449375.  
15

Reference may even be made to Spngstad *et al* (1995 Plant Cell Tissue Organ Culture  
40 pp 1-15) as these authors present a general overview on transgenic plant construction.

20 In one embodiment, the plant is a grapevine. There are a number of teachings in the art on how to prepare transformed grapevines. For example, reference may be made to Baribault *et al* (J Exp Bot 41 (229) 1990 1045-1050), Baribault *et al* (Plant Cell Rep 8 (3) 1989 137-140), Scorza *et al* (J Am Soc Horticultural Science 121 (4) 1996 616-619), Kikkert *et al* (Plant Cell Reports 15 (5) 1996 311-316), Golles *et al* (Acta  
25 Hortic 1997 vol 447 Number: Horticultural Biotechnology in Vitro Culture and Breeding Pages 265-275), Gray and Scorza (WO-A-97/49277) and Simon Robinson *et al* (Conference abstracts and paper presented in Biotechnology - Food and Health for the 21st Century, Adelaide, Australia, 1998). By way of example Robinson *et al* (*ibid*) disclose a method for transforming grapevine wherein somatic embryos are  
30 induced on callus formed from another tissue and *Agrobacterium* infection is used to transfer target genes into the embryo tissue.

Further reference may be made to the teachings of Andrew Walker in Nature Biotechnology (Vol 14, May 1996, page 582) who states that:

5       *"The grape, one of the most important fruit plants in the world, has been difficult to engineer because of its high levels of tannins and phenols, which interfere with cell culture and transformation; the compounds oxidize quickly and promote the decay of grape cells."*

10       In that same edition of Nature Biotechnology, Perl *et al* (pages 624-628) report on the use of the combination of polyvinylpolypyrrolidone and dithiothreitol to improve the viability of grape transformation during *Agrobacterium* infection.

15       Hence, the present invention provides an alternative means for transforming grape. In this regard, the antioxidant that is prepared *in situ* by a grape transformed in accordance with the present invention improves the viability of grape transformation during *Agrobacterium* infection.

20       Thus, according to one aspect of the present invention, there is provided the use of an antioxidant prepared *in situ* in order to effectively transform a grape.

25       In some instances, it is desirable for the recombinant enzyme or protein to be easily secreted into the medium to act as or to generate an anti-oxidant therein. In such cases, the DNA encoding the recombinant enzyme is fused to *inter alia* an appropriate signal sequence, an appropriate promoter and an appropriate terminator from the chosen host.

30       For example, for expression in *Aspergillus niger* the *gpdA* (from the Glyceraldehyde-3-phosphate dehydrogenase gene of *Aspergillus nidulans*) promoter and signal sequence is fused to the 5' end of the DNA encoding the mature lyase. The terminator sequence from the *A. niger* *trpC* gene is placed 3' to the gene (Punt, P.J. et al 1991 - (1991): J. Biotech. 17, 19-34). This construction is inserted into a vector containing a replication origin and selection origin for *E. coli* and a selection marker

for *A. niger*. -Examples of selection markers for *A. niger* are the amdS gene, the argB gene, the pyrG gene, the hygB gene, the BmlR gene which all have been used for selection of transformants. This plasmid can be transformed into *A. niger* and the mature lyase can be recovered from the culture medium of the transformants.

5 Eventually the construction could be transformed into a protease deficient strain to reduce the proteolytic degradation of the lyase in the medium (Archer D.B. et al 1992 -Biotechnol. Lett. 14, 357-362).

10 In addition, and as indicated above, aside from using *Aspergillus niger* as the host, there are other industrial important microorganisms which could be used as expression systems. Examples of these other hosts include: *Aspergillus oryzae*, *Aspergillus sp.*, *Trichoderma sp.*, *Saccharomyces cerevisiae*, *Kluyveromyces sp.*, *Hansenula sp.*, *Pichia sp.*, *Bacillus subtilis*, *B. amyloliquefaciens*, *Bacillus sp.*, *Streptomyces sp.* or *E. coli*.

15 In accordance with the present invention, a suitable marker or selection means may be introduced into the host that is to be transformed with the nucleotide sequence. Examples of suitable markers or selection means are described in any one of WO-A-93/05163, WO-A-94/20627, GB patent application No. 9702591.0 (filed 7 February 1997), GB patent application No. 9702576.1 (filed 7 February 1997), GB patent application No. 9702539.9 (filed 7 February 1997), GB patent application No. 9702510.0 (filed 7 February 1997) and GB patent application No. 9702592.8 (filed 7 February 1997).

20

25 In summation, the present invention relates to a process comprising preparing a medium that comprises an anti-oxidant and at least one other component, the process comprising preparing *in situ* in the medium the anti-oxidant; and wherein the anti-oxidant is prepared from a glucan by use of recombinant DNA techniques and/or the anti-oxidant is prepared by use of a recombinant glucan lyase.

In a preferred-embodiment, the present invention relates to a process a process of preparing a medium that comprises an anti-oxidant and at least one other component, the process comprising preparing *in situ* in the medium the anti-oxidant; and wherein the anti-oxidant is prepared from a glucan by use of a recombinant glucan lyase.

5

In a more preferred embodiment, the present invention relates to a process of preparing a medium that comprises an anti-oxidant and at least one other component, the process comprising preparing *in situ* in the medium the anti-oxidant; wherein the anti-oxidant is prepared from a glucan by use of a recombinant glucan lyase; and

10

wherein the anti-oxidant is anhydro-fructose.

The present invention will now be described only by way of example.

#### TRANSGENIC GRAPE

15

Transformed grapes are prepared following the teachings of Perl *et al* (*ibid*) but wherein the use of the combination of polyvinylpolypyrrolidone and dithiothreitol is optional. In these studies, the grapes are transformed with any one of the nucleotide sequences presented as SEQ ID No. 7-12. The transformation leads to *in situ* preparation of 1,5-D-anhydrofructose. The transformed grapes are beneficial for one or more of the reasons mentioned earlier.

20

Details on these studies are as follows.

#### 25 Tissue-culture systems for transformation studies

The long term somatic embryogenic callus culture is developed from the vegetative tissues of anthers of *Vitis vinifera* CV Superior Seedless. Methods for another culture, induction of somatic embryogenesis and maintenance of embryogenic cultures, are previously described (Perl *et al*, 1995, Plant Sci **104**: 193-200). Briefly,

30

embryogenic calli are maintained on solidified (0.25% gelrite) MS medium (Murashige and Skoog, 1962, Physiol Plant **15**: 473-497) supplemented with 6%

sucrose, 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 5 mg/L Indole-3-aspartic acid (IASP), 0.2 mg/L 6-benzyladenine (BAP) and 1 mg/L abscisic acid (ABA). Proembryogenic calli are induced by transferring the calli to MS medium supplemented with the same phytohormones, but 2,4-D is substituted with 2 mg/L 2-naphthoxyacetic acid (NOA). This stage is used for transformation experiments.

### *Agrobacterium* strains

For studying the sensitivity of grape embryogenic calli to the presence of different *Agrobacterium* strains, or for stable transformation experiments, cocultivation is attempted using the following *A tumefaciens* strains: EHA 101-p492 (Perl *et al.*, 1993, Bio/Technology **11**:715-718); LBA 4404-pGPTV (Becker *et al.*, 1992, Plant Mol Biol **20**: 1195-1197); and GVE 3101-pPCV91 (Vancanneyt *et al.*, 1990, Mol Gen Genet **220**: 245-250). These strains contain the binary vectors conferring resistance to kanamycin (*nptII*), basta (*bar*) and hygromycin (*hpt*), respectively, all under the control of the nopaline-synthase (NOS) promoter and terminator. Bacteria are cultured with the proper antibiotics in liquid LB medium for 24 hours at 28°C at 200 rpm.

### Cocultivation

For studying the sensitivity of grape embryogenic calli to different *Agrobacterium* strains, bacterial cultures with different optical densities (0.1-0.7 at 630 nm) are prepared from an overnight culture of *Agrobacterium* strains. Bacteria are centrifuged 5 minutes, 5000 rpm and resuspended in antibiotic free McCown's Woody Plant Medium (WPM) (Lloyd and McCown, 1981, Int Plant Prop Soc Proc **30**: 421-427). Three grams fresh weight of embryogenic calli (7 days after transfer to NOA containing medium) are resuspended in 10 ml of overnight cultured bacterial suspensions for 5 minutes, dry blotted and transferred to Petri dishes containing regeneration medium [basal WPM medium supplemented with thidiazuron (TDZ) (0.5 mg/L), Zeatin riboside (ZR) (0.5 mg/L), and sucrose (3%)]. The regeneration medium is solidified with gelrite (0.25% w/v) and the calli, after initial drainage of excess bacteria, are cocultivated in the dark at 25°C for different times (5 minutes

up to 7 days). For stable transformation experiments, inoculum (OD 0.6 at 630 nm) is prepared from an overnight culture of LBA 4404 or GVE 3101. Bacteria are centrifuged 5 minutes, 5000 rpm and resuspended in antibiotic-free WPM medium. Embryogenic calli (3g fresh weight) are resuspended in 10 ml of bacteria for 5 minutes, dry blotted and transferred to Petri dishes containing solidified (0.25% w/v) gelrite regeneration medium supplemented with different antioxidants. The calli are cocultivated for 48 hours in the dark at 25°C.

### Selective culture

Following 48 hours of cocultivation, the embryogenic callus is maintained in the dark for 7 days on antioxidant containing regeneration medium. Subsequently, the calli are collected on a sterile metal screen and transferred to fresh WPM regeneration medium at 25°C under 40  $\mu\text{E}/\text{m}^2/\text{s}$  (white fluorescent tubes). All regeneration media are supplemented with 400 mg/L claforan, 1.5 g/L malt extract and different selectable markers: kanamycin (50-500 mg/L), hygromycin (15 mg/L) and Basta (1-10 mg/L). Periodic increases in hygromycin concentration are used. The putative transformed calli are cultured on regeneration medium supplemented with 15 mg/L hygromycin. Every two weeks the regenerating calli are transferred to fresh medium supplemented with 20 and 25 mg/L hygromycin respectively. Control, untransformed grape calli are also cultured on selective media and are periodically exposed to increasing hygromycin concentrations. Green adventitious embryos, which developed on calli cultured for 8-10 weeks on selective regeneration medium, are transferred to germination medium. Embryo germination, rooting and subsequent plantlet development are induced on WPM as described (Perl *et al*, 1995, Plant Sci **104**: 193-200), supplemented with 25 mg/L hygromycin or 10 mg/L basta. Conversion of vitrified abnormal plantlets into normal-looking grape plantlets are obtained using solidified WPM medium supplemented with 0.1 mg/L NAA as described (Perl *et al*, 1995, Plant Sci **104**: 193-200).

## TRANSGENIC POTATOES

General teachings on potato transformation may be found in our copending patent applications PCT/EP96/03053, PCT/EP96/03052 and PCT/EP94/01082 (the contents  
5 of each of which are incorporated herein by reference).

For the present studies, the following protocol is adopted.

### Plasmid construction

10

The disarmed *Agrobacterium tumefaciens* strain LBA 4404, containing the helper *vir* plasmid pRAL4404 (Hoekema *et al*, 1983 Nature **303** pp 179-180), is cultured on YMB agar ( $K_2HPO_4 \cdot 3H_2O$  660 mg l<sup>-1</sup>,  $MgSO_4$  200 mg l<sup>-1</sup>, NaCl 100 mg l<sup>-1</sup>, mannitol 10 g l<sup>-1</sup>, yeast extract 400 mg l<sup>-1</sup>, 0.8% w/v agar, pH 7.0) containing 100 mg l<sup>-1</sup>  
15 rifampicin and 500 mg l<sup>-1</sup> streptomycin sulphate. Transformation with pVICTOR IV GNG E35S *nagB* IV2' or pVICTOR IV GNG *rbc nagB* IV2' or pVICTOR IV GNG E35S *nagB*' (which correspond to each of pVICTOR IV GNG E35S *nagB* IV2 or pVICTOR IV GNG *rbc nagB* IV2 or pVICTOR IV GNG E35S *nagB* but wherein each of those plasmids also contains any one of the nucleotide sequences shown as  
20 SEQ ID No.s. 7-12 operatively linked to a functional promoter) is accomplished using the freeze-thaw method of Holters *et al* (1978 Mol Gen Genet **163** 181-187) and transformants are selected on YMB agar containing 100 mg l<sup>-1</sup> rifampicin and 500 mg l<sup>-1</sup> streptomycin, and 50 mg l<sup>-1</sup> gentamycin sulphate.

### 25 Transformation of plants

Shoot cultures of *Solanum tuberosum* cv Saturna are maintained on LS agar containing Murashige Skoog basal salts (Sigma M6899) (Murashige and Skoog, 1965, Physiol Plant **15** 473-497) with 2  $\mu$ M silver thiosulphate, and nutrients and vitamins  
30 as described by Linsmaier and Skoog (1965 Physiol Plant **18** 100-127). Cultures are maintained at 25°C with a 16h daily photoperiod. After approximately 40 days, subculturing is performed during which leaves are removed, and the shoots cut into

mononodal segments of approximately 8 mm length.

Shoot cultures of approximately 40 days maturity (5-6 cm height) are cut into 8 mm internodal segments which are placed into liquid LS-medium containing  
5 *Agrobacterium tumefaciens* transformed with pVICTOR IV GNG E35S *nagB* IV2' or pVICTOR IV GNG *rbc nagB* IV2' or pVICTOR IV GNG E35S *nagB*' ( $A_{660} = 0.5$ , pathlength 1 cm). Following incubation at room temperature for 30 minutes, the segments are dried by blotting on to sterile filter paper and transferred to LS agar (0.8% w/v containing 2 mg l<sup>-1</sup> 2,4-D and 500 µg l<sup>-1</sup> trans-zeatin. The explants are  
10 covered with filter paper, moistened with LS medium, and covered with a cloth for three days at 25°C. Following this treatment, the segments are washed with liquid LS medium containing 800 mg l<sup>-1</sup> carbenicillin, and transferred on to LS agar (0.8% w/v) containing 1 mg l<sup>-1</sup> trans-zeatin, 100 µg l<sup>-1</sup> gibberellic acid (GA3), with sucrose (eg 7.5 g l<sup>-1</sup>) and glucosamine (eg 2.5 g l<sup>-1</sup>) as the selection agent.

15 The segments are sub-cultured to fresh substrate each 3-4 weeks. In 3 to 4 weeks, shoots develop from the segments and the formation of new shoots continues for 3-4 months.

#### 20 Rooting of regenerated shoots

The regenerated shoots are transferred to rooting substrate composed of LS-substrate, agar (8 g/l) and carbenicillin (800 mg/l).

25 The transgenic plants may be verified by performing a GUS assay on the co-introduced  $\beta$ -glucuronidase gene according to Hodal, L. *et al.* (Pl. Sci. (1992), 87: 115-122).

Alternatively, the transgenic genotype of the regenerated shoot may be verified by  
30 performing NPTII assays (Radke, S. E. *et al.*, Theor. Appl. Genet. (1988), 75: 685-694) or by performing PCR analysis according to Wang *et al* (1993, NAR 21 pp 4153-4154).

### Transfer to soil

The newly rooted plants (height approx. 2-3 cms) are transplanted from rooting substrate to soil and placed in a growth chamber (21°C, 16 hour light 200-400uE/m<sup>2</sup>/sec). When the plants are well established they are transferred to the greenhouse, where they are grown until tubers had developed and the upper part of the plants are senescing.

### Harvesting

The potatoes are harvested after about 3 months.

## **TRANSGENIC MAIZE PLANTS**

### **Introduction**

Since the first publication of production of transgenic plants in 1983 (Leemans, 1993 Biotechnology 11 s22), there have been numerous publications of production of transgenic plants including especially dicotyledon crop plants.

Until very recently there are very few reports on successful production of transgenic monocotyledonary crop plants. This relatively slow development within monocots are due to two causes. Firstly, until the early 1980s, efficient regeneration of plants from cultured cells and tissues of monocots had proven very difficult. This problem is ultimately solved by the culture of explants from immature and embryogenic tissue, which retain their morphogenic potential on nutrient media containing plant growth regulators. Secondly, the monocots are not a natural host for *Agrobacterium tumefaciens*, meaning that the successful developed techniques within the dicots using their natural vector *Agrobacterium tumefaciens* is unsuccessful for many years in the monocots.

Nevertheless, it is now possible to successfully transformation and produce fertile

transgenic plants of maize using methods such as: (1) Silicon Carbide Whiskers; (2) Particle Bombardment; (3) DNA Uptake by PEG treated protoplast; or (4) DNA Uptake in Electroporation of Tissue. Each of these methods - which are reviewed by Thompson (1995 Euphytica 85 pp 75-80) - may be used to prepare *inter alia* transgenic maize according to the present invention.

In particular, the particle Gun method has been successfully used for the transformation of monocots. However, EP-A-0604662 reports on a different method of transforming monocotyledons. The method comprises transforming cultured tissues of a monocotyledon under or after dedifferentiation with *Agrobacterium* containing a super binary vector as a selectable marker a hygromycin-resistant gene is used. Production of transgenic calli and plant is demonstrated using the hygromycin selection. This method may be used to prepare *inter alia* transgenic maize according to the present invention.

Subsequent to the method of EP-A-0604662, EP-A-0672752 reports on non-dedifferentiated immature embryos. In this regard, both hygromycin-resistance and PPT-resistance genes are used as the selectable marker, with PPT giving rise to 10% or more independent transformed plants. This method may be used to prepare *inter alia* transgenic maize according to the present invention.

To date, it would appear that transgenic maize plants can be successfully produced from easily-culturable varieties - such as the inbred line A188. In this regard, see the teachings of Ishida *et al* (1996 Nature Biotechnology 14 pp 745-750). The method disclosed by these workers may be used to prepare *inter alia* transgenic maize according to the present invention.

Vasil (1996 Nature Biotechnology 14 pp 702-703) presents a further review article on transformation of maize. Even though it is possible to prepare transformed maize by use of, for example, particle Gun mediated transformation, for the present studies the following protocol is adopted.

### Plasmid construction

The disarmed *Agrobacterium tumefaciens* strain LBA 4404, containing the helper *vir* plasmid pRAL4404 (Hoekema *et al*, 1983 Nature **303** pp 179-180), is cultured on  
5 YMB agar ( $K_2HPO_4 \cdot 3H_2O$  660 mg l<sup>-1</sup>,  $MgSO_4$  200 mg l<sup>-1</sup>, NaCl 100 mg l<sup>-1</sup>, mannitol 10 g l<sup>-1</sup>, yeast extract 400 mg l<sup>-1</sup>, 0.8% w/v agar, pH 7.0) containing 100 mg l<sup>-1</sup> rifampicin and 500 mg l<sup>-1</sup> streptomycin sulphate. Transformation with pVICTOR IV GNG E35S *nagB* IV2' or pVICTOR IV GNG *rbc nagB* IV2' or pVICTOR IV GNG E35S *nagB*' is accomplished using the freeze-thaw method of Holters *et al* (1978 Mol  
10 Gen Genet **163** 181-187) and transformants are selected on YMB agar containing 100 mg l<sup>-1</sup> rifampicin and 500 mg l<sup>-1</sup> streptomycin, and 50 mg l<sup>-1</sup> gentamycin sulphate.

### Isolation and cocultivation of explants

15 Immature embryos of, for example, maize line A188 of the size between 1.5 to 2.5 mm are isolated and cocultivated with *Agrobacterium tumefaciens* strain LBA 4404 in N6-AS for 2-3 days at 25°C under illumination. Thereafter, the embryos are washed with sterilized water containing 250 mg/l of cefotaxime and transferred to an LS medium and 250 mg/l cefotaxime and glucosamine in concentrations of up to 100  
20 mg/l (the medium is hereafter called LSS1).

### Conditions for the selection of transgenic plants

The explants are cultured for three weeks on LSS1 medium and then transferred to  
25 an LS medium containing glucosamine and cefotaxime. After three weeks on this medium, green shoots are isolated.

### Rooting of transformed shoots

30 Transformed shoots are transferred to an MS medium containing 2 mg/l for rooting. After four weeks on this medium, plantlets are transferred to pots with sterile soil for acclimatisation.

Transformation of guar cotyledonary explants is performed according to Joersbo and Okkels (PCT/DK95/00221) using *Agrobacterium tumefaciens* LBA4404 harbouring a suitable plasmid.

10 Other modifications of the present invention will be apparent to those skilled in the art.